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A modular 'universal' TaqMan™ assay

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Introduction

The 5′ exonuclease (TaqMan™) assay produces a direct proportional readout for the progress of PCR reactions. It involves the displacement and cleavage of a fluorogenic oligonucleotide probe which hybridizes to a region between the primers directing a PCR. This has the advantage that specificity is determined by three independent oligonucleotides, but the disadvantage that a different, complex and expensive TaqMan probe is required for each PCR reaction to be quantitated. A method is described by which a 'universal' TaqMan probe can be used to quantitate almost any PCR reaction through the modular assembly of an assay site at one end of the amplicon. The only specific requirement for each reaction is that a modified PCR primer must be synthesised with an additional 20 nucleotides at its 5′ end.

The TaqMan™ assay permits the assessment of the progress of a PCR reaction by the displacement and cleavage of a fluorogenic probe hybridizing to the middle of an amplicon (Ref. 1). The probe comprises about 20 nucleotides, with the 5′ end substituted with a fluorophore (Tet) and the 3′ end with a quencher (Tamra). The 3′ end is also phosphorylated to prevent it acting as a primer. As chain elongation encounters the annealed probe, it is displaced and cleaved by the 5′ exonuclease activity of the Taq polymerase, decoupling the fluorophore from the quencher and leading to an increase in fluorescence. The cleavage is stoichiometrically related to the amplification process and, provided that there are no cross-reactions, it can be used as quantitative data without the need for post-PCR analytical processes, such as electrophoresis.

The method has the additional advantage that specificity is determined by three oligonucleotides, but the disadvantage that costly TaqMan probes must be synthesised for each amplicon.

Jeffreys has introduced the concept of TAGed primers for PCR reactions (Ref. 2, 3). These have a number of applications and advantages (Ref. 4). This paper describes their use as the target for a modular extension of amplicons to include a standard TaqMan target sequence and, hence, permit the use of a standard probe and set of additional primers to produce a 'universal' TaqMan readout system ('TAGMan').

Design principles

PCR primers with a 5′ TAG lead to the inclusion of a foreign and arbitrarily chosen TAG sequence at one (or both) end(s) of amplicons. This sequence can be directly used as a priming target or for the addition of an extension (Fig. 1). The latter can include a region of sequence identical to that of a TaqMan probe, flanked on the 3′ end by a copy of the original TAG sequence and by a different TAG sequence at the 5′ end. Amplicons which incorporate the additional sequence are amplified by the provision of a driving concentration of the distal TAG primer. The TaqMan probe sequence must be suitably spaced from the 3′ end of the driving TAG primer and, in these experiments, is a direct copy of the sequence of the TagMan probe. Thus, the hybridization target for the probe is produced as a result of copying the extended amplicon and is absent from the initial reactants.

Table 1 shows the primers used in the experiments illustrated here.

PCR conditions

Reactions were carried out in a 50 ml volume in Treff 0.5 ml microfuge tubes in:

10 mmol/l TRIS-HCl (pH 8.8 at 25°C)
1.5 mmol/l MgCl2

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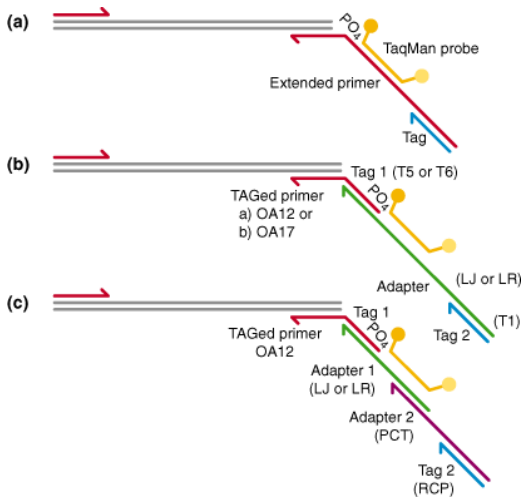

FIGURE 1. Reaction schema for the TAGMan assay. (a) A simple approach using a very long 5′ extension of the template-specific primer that directly incorporates the TAGMan probe site and the site for the driving TAG primer. (b) A modular approach in which the template-specific primer incorporates only a fairly short (20 bases) 5′ TAG 1 site, through which a long standard adapter incorporates both the TAGMan probe site and the diving TAG 2 site. It is this latter reaction scheme which was used in these experiments, but in initial experiments the probe was located too close to the driving TAG. Therefore, (c) a secondary adapter was used to shift the final priming site to a more distal location.

50 mmol/l KCl
1% Triton X-100 buffer (Bioline) or 16 mmol/l (NH4)2SO4
67 mmol/l TRIS-HCl (pH 8.8 @ 25°C)
0.1% Tween-20

together with 50 mmol/l final each dNTP and 10–50 ng genomic template DNA. The standard final primer concentration was 0.3 mmol/l, although in many cases the concentrations of the initiating and extending primers was very much lower (3–30 nmol/l). Evaporation was prevented by the addition of a drop of light mineral oil (Sigma). The reaction mixtures were boiled for 5 minutes, then cooled to 65°C or 80°C before addition of 0.25 units of Taq polymerase (Bioline).

Typical reaction conditions were 66°C for 1 minute, 72°C for 1 minute, 93°C for 1 minute × 35 in a MJ Research PTC 100 (medium fast) thermal cycler. Some experiments with low concentrations of initial primer had a preliminary six cycles of annealing for 10 minutes, followed by 35 1-minute-per-phase cycles.

Fluorescence readout

Table 2 and Table 3 show the relationship between fluorescence and band intensity for a number of TAGMan

Table 1. Sequences of primers used in these experiments

Name	Sequence	Correspondences	
Allele-specific primer	OA17	ACGCTTGCGAATGGCGAGATCCAGAAGAAGACTGTAGGCA	T6-C6 exon 15
Adapter 1	LJ	AGGCCTGGTACCTGCGTACTGAACCTGCCTGTCACCCAGAT	
		CACCGTGTCGAGGTCGTCGCT	T1-InsProbe-T5
	LR	AGGCCTGGTACCTGCGTACTATCAGAACCTGCCTGTCACCC	
		AGATCACGCTTGCGAATGGCGAGAT	T1-InsProbe-T6
Adapter 2	PCT	CGTACGAACGGTTACGATTCAGGCCTGGTACCTGCGTACT	(RCP-T1)
TAGs	T1	AGGCCTGGTACCTGCGTACT	
	RCP	CGTACGAACGGTTACGATTC	
	T4	CGTCGAGTCCAGCAACCTGC	
	T5	ACCGTGTCGAGGTCGTCGCT	
	T6	ACGCTTGCGAATGGCGAGAT	
Genomic primer	MH 27	TGGTTACACTAGACTGGTTTTCCC	Location: C6 intron 15

The sequences are given 5′ to 3′ and the column entitled correspondences is a short guide to their relationships.

Table 2. TAGMan reactions Experiment with large amount (~1 ng) of preformed template ready for extension

Template	Adapter 1	Adapter 2	Driving primers	Fluorescence			PCR	Correlation	
				Tet	Tamra	Ratio	Meaning	product	
none	-	-	T4 & T5	207	86	2.4	-	-	OK
Preformed amplicon	- /	-	T4 & T5	263	295	2.77	-	+	OK (control)
none	LJ	PCT	T4 & RCP	359	105	3.42	(+)	-	unclear
Preformed amplicon	LJ	PCT	T4 & RCP	584	106	5.51	++	++	OK

Preformed amplicon: dimer of: OA11 (CGTCGAGTCCAGCAACCTGC GGATGTGGAATGCCAATG) and OA12 (ACCGTGTCGAGGTCGTCGCT TTGCCCAGTTCTCACAT), comprising T4 – gene sequence – T5 (i.e. CGTCGAGTCCAGCAACCTGC-GGATGTGGAATGCCAATGTGAGAGAACTGGGCAAAGCGACGCACCTCGACACGGT).

reactions. The correlations are generally good, which provides evidence that the approach is feasible. No claim is made from these data that the method is fully quantitative.

Initial experiments using an extender with a small interval between the last nucleotide of the driving TAG site failed. The exact spacing requirement between primer and TaqMan probe has not been determined, but seven bases are sufficient and three are too few.

Table 3 shows data from a stringent test of the method, the detection of a single-base allelic difference between two homozygous human genomic DNA templates. Although the reactions produced only weak bands on gel analysis, the fluorescent readout was clearly above background.

Conclusion

The TAGMan method offers a reasonably convenient method for the economical conversion of most PCR reactions to fluorogenic readout using common extender and

Table 3. TAGMan reactions Experiment to distinguish a single base-change variant directly from genomic DNA using OA17 allele-specific primer

Template	Adapter 1	Adapter 2	Driving primers	Fluorescence				PCR product	Correlation
				Tet	Tamra	Ratio	Meaning		
None	LR		MH27 & T1	215	116	1.86	-	-	OK
Positive genome	LR		MH27 & T1	292	119	2.45	(+)	(+)	OK weak +
Negative genome	LR		MH27 & T1	234	125	1.87	-	-	OK
None	LR	PCT	MH27& RCP	241	129	1.86	-	-	OK
Positive genome	LR	PCT	MH27& RCP	279	129	2.15	(+)	(+)	OK weak +
Negative genome	LR	PCT	MH27& RCP	214	117	1.82	-	-	OK

The results of a very demanding test, in which a single base (T/C) allelic difference in two genomic DNA samples was investigated using a primer for the C allele.

TAG primers and a common TaqMan probe. It can be used in reactions where the template-specific primers are close together (or even overlapping).

Its disadvantages are that the specificity enhancement possible by the use of an included TaqMan probe is lost and there are potential pitfalls in the use of more and larger primers in a more complicated reaction.

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Products Used

Triton X-100: Triton X-100 from Stratagene
Triton X: Triton X from Sigma
triton X-100: triton X-100 from Promega Corporation
light mineral oil: light mineral oil from Sigma
Taq DNA polymerase: Taq DNA polymerase from PE Applied Biosystems
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Promega Corporation
Taq polymerase: Taq polymerase from Boehringer Mannheim
Taq polymerase: Taq polymerase from Pharmacia
Taq polymerase: Taq polymerase from Bioline
Taq polymerase: Taq polymerase from Advanced Biotechnologies
Taq polymerase: Taq polymerase from Boehringer Mannheim
Taq polymerase: Taq polymerase from Bioline
Thermal Cycler: Thermal Cycler from Techne (Cambridge) Ltd
thermal cycler: thermal cycler from MJ Research Inc